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A developing novel alternative bio-oxidation approach to treat low-grade refractory sulfide ores at circumneutral pH

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The extensive neutralization required in acidic bio-oxidation, a conventional pretreatment for low-grade refractory matrices in the gold industry, constitutes one of the principal drawbacks due to the large volume of waste streams. Performing an oxidative pretreatment at circumneutral pH with an *in-situ* neutralization would avoid the production of undesirable waste, causing potential economic and environmental advantages. For the first time, this investigation evaluates a novel process involving a biological oxidative pretreatment for low-grade refractory ore using two biosafety level 1 neutrophilic microorganisms encompassing *Thiobacillus thioeparus* and *Starkeya novella* at near-neutral pH. Optimal bacterial growth conditions were determined regarding the culture medium and initial energy source using UV-visible and manual cell counting (cells/mL). Thereafter, biological oxidation of different matrices, including first elemental sulfur and subsequently a refractory sulfidic ore, was evaluated in batch flask cultures and then scaled up into a bioreactor using optimal experimental conditions. Results revealed that culture media containing ca. 4.5 and ca. 0.9 g/L thiosulfate favored biological oxidation of the refractory sulfidic ore using *T. Thioparus* and *S. Novella*, respectively, which led to corresponding sulfide oxidation of 27 and 14% within 10 days, comparable to reported studies. The biological action was confirmed by C/S detector and SEM technique of pre- and post-pretreatment residues. Overall, this research is a step forward to advance the understanding of a biological pretreatment out of the highly acidic pH range, promoting the view of a net-zero target by potentially reducing the production of more significant waste streams compared to conventional operations.

KEYWORDS

bio-oxidation, low-grade refractory ores, neutrophiles, neutral oxidation, sulfur removal, biohydrometallurgy

Introduction

A huge amount of hazardous waste e.g., cyanide bearing tailings and residues, along with additional operational costs could arise from direct processing of low-grade refractory gold ores without an oxidative pre-treatment (Marsden and House, 2006; Adams, 2016). The processing of low-grade refractory gold ores is a growing challenge in the industry as the average grade of deposits is constantly decreasing over time, which might include matrices with increasingly complex mineralogy associated with carbonaceous materials (Marsden and House, 2006; Adams, 2016). Currently, one-third of the total global gold production is obtained from refractory deposits, which require an oxidative pre-treatment to extract gold economically (Marsden and House, 2006; Yang et al., 2013).

The principal pre-treatment methods to oxidize refractory gold ores include roasting, pressure oxidation, and bio-oxidation. Roasting and pressure oxidation are effective technologies but have high capital and operating cost associated with increased energy input and emission of greenhouse gases and can be unprofitable for refractory ores with extremely low grades (Azizitorghabeh et al., 2022; Darvanjooghi et al., 2022). Bio-oxidation has several advantages over traditional methods owing to its less energy requirements and environmental impact by decreasing gas emissions and can be suitable for low-grade matrices (Aswegen et al., 2007; Kaksonen et al., 2014b). Bio-oxidation is a biological oxidation technique involving acidophilic bacteria to enhance the extraction of valuable metals by accelerating the oxidation of the sulfide matrix in refractory ores, which in turn improves gold recovery during cyanide leaching. Biological oxidative technologies encompassing BIOX[®] and BacTech Bacox have been successfully executed in the gold industry (Darvanjooghi et al., 2022), rendering uneconomic gold reserves into valuable materials which would otherwise be considered unprofitable resources owing to their low grade and generation of more production-related waste.

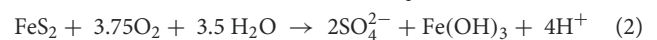
Despite the efficacy of bio-oxidation employing acidophilic bacteria, pH control endures as one of the main drawbacks as a consequence of significantly acidic pH conditions and extensive neutralization of effluents afterwards. In standard bio-oxidation plants, pH control accounts for ca. 30% of the total operational cost comprising sulfuric acid, lime, and limestone (Aswegen et al., 2007; Lee et al., 2021). Prior to cyanide leaching at alkaline pH, neutralizing reagents, e.g., slaked lime and limestone, are applied to generate effluents with near-neutral pH and avoid acid mine drainage (AMD) (Darvanjooghi et al., 2022). Moreover, highly acid pH operations could have more risk of leaving residual acid in cyanide leaching, which could lead to cyanide release as HCN (Lee et al., 2021). Hence, conducting an oxidative pretreatment out of the extreme acidic pH range would prevent the generation of undesirable waste streams and diminish the concentration of reagents required in the neutralization stage, bringing potential economic and environmental advantages.

A bio-oxidation pretreatment performed out of the extreme acidic pH zone must contemplate neutrophilic sulfur-oxidizing bacteria instead of acidophilic microorganisms. Neutrophilic bacteria include microorganisms that can thrive at pH > 4 but have a more favorable growth at a circumneutral pH between pH 6.0 and 8.0 (Kelly et al., 2015; Lee et al., 2021). Most neutrophiles are commonly found in soils, wastewater, and marine freshwater and have an important role in the natural sulfur cycle on earth (McNeice et al., 2022). Neutrophilic bacteria also have significant importance in the waste remediation industry, including desulfurizing both liquid and gas waste streams.

Most of the strains investigated includes but are not limited to *Citrobacter*, *Halothiobacillus*, *Nocardioides*, *Parapedobacter*, *Rhodobacter*, *Rhodopseudomonas*, *Starkeya*, *Thiobacillus*, *Thermithiobacillus*, and *Thiomonas*. Recent research has emphasized the efficacy of neutrophilic microorganisms in oxidizing reduced sulfur species, especially in gaseous waste streams such as hydrogen sulfide (H₂S) which is preferentially oxidized to sulfate in batch experiments (Lee et al., 2021). The effectiveness of the desulfurization of H₂S with neutrophilic bacteria has led to its industrial implementation in H₂S odor control in industrial waste encompassing BIOREM technologies Inc. and ALKEN

ENZ-ODOR[®] 8 Alken-Murray Corporation (Alken-Murray, 2006; Biorem Technology Inc, 2015). Nonetheless, implementing neutrophilic bacteria in mining has been scarcely explored, and require further research.

Until now, most research has comprised bio-oxidation with acidophilic bacteria at low pH, which is based on the oxidation of reduced sulfur species as well as ferric iron through contact and non-contact biological mechanisms (Azizitorghabeh et al., 2022). The overall bio-oxidation reaction of pyrite using acidophilic bacteria is displayed in Equation 1, where Fe³⁺ is regenerated throughout the reaction by iron-oxidizing bacteria, which is unlikely to take place at circumneutral pH due to Fe insolubility at pH > 4 (Percak-Dennett et al., 2017). Scarce research toward performing a bio-oxidation operation with neutrophilic bacteria applied to low-grade refractory sulfidic ores has been reported. Microbial oxidation of pyrite at circumneutral pH has been suggested to occur through a polythionate intermediate model and/or sorbed Fe redox cycling model (Moses et al., 1987; Moses and Herman, 1991; Schippers, 2004). The overall reaction for microbially assisted reaction in natural environments is shown in Equation 2, which assumed a rapid formation of iron (III) hydroxide (Fe(OH)₃) due to iron insolubility.



Few research groups have reported the dissolution of solid sulfide matrices with neutrophilic bacteria, primarily using a solid-to-liquid ratio <5 g/L. Vázquez-Rodríguez et al. (2015) evaluated *T. thioparus* in the dissolution of mercury sulfide (HgS), and Percak-Dennett et al. (2017) assessed indigenous microorganisms including *Rhizobiales* and *Ralstonia* species in synthetic pyrite (FeS₂) oxidation. Nonetheless, to our knowledge, there is no direct investigation into the oxidative capacities of neutrophilic bacteria on the bio-oxidation of low-grade refractory sulfidic ores at initial circumneutral pH and high pulp densities.

This paper assesses a new technological opportunity evaluating the oxidative capacity of two neutrophilic bacteria with refractory sulfidic ore. First, the effect of medium composition on bacterial growth was evaluated by changing the individual concentration of certain ingredients in the culture medium to find optimal growth conditions for bio-oxidation tests. Second, elemental sulfur was selected as a baseline test to confirm the oxidative abilities of both microorganisms on solid matrices. Third, bio-oxidation tests were performed using a low refractory sulfidic ore by modifying experimental conditions and later scaling up in a 1L jacketed bioreactor. Lastly, a comparative study with similar investigations was provided highlighting the output of this research.

Materials and methods

Sample preparation

Elemental sulfur (S⁰) with a purity of 99.5% (Alfa Aesar) and particle size <44 μm was initially used in bio-oxidation tests. Subsequently, a low-grade refractory sulfidic ore from a Canadian deposit was employed in further bio-oxidation experiments. The ore

TABLE 1 Modified composition of TM medium used with *S. novella*^a.

Medium	Na ₂ S ₂ O ₃ ·5H ₂ O (g/L)	Yeast extract (g/L)	Glucose (g/L)
TM1	10	0.3	1.5
TM2	5	0.3	1.5
TM3	2	0.3	1.5
TM4	10	5	1.5
TM5	5	5	1.5
TM6	2	5	1.5

^aRest of the ingredients in media TM1–6 are the same found in standard TM, including 0.1 g/L (NH₄)₂SO₄, 4 g/L K₂HPO₄, 4 g/L KH₂PO₄, 0.1 g/L MgSO₄·7H₂O, 0.1 g/L CaCl₂, 0.02 g/L FeCl₃·6H₂O, and 0.02 g/L MnSO₄·H₂O.

was acquired in a coarse form and later crushed and pulverized into fine particles. Manual riffers were used to obtain representative samples. The particle size of 80% passing (P₈₀) of the sample prior to bio-oxidation was measured with laser particle size analyzer (Malvern Mastersizer 3000).

Growth of neutrophilic bacteria

Thiobacillus Thioparus (DSMZ 505) and *Starkeya Novella* (DSMZ 506) were acquired in freeze-dried form from The German Collection of Microorganism and Cell Cultures (DSMZ). Both neutrophilic microorganisms are classified as biosafety level 1 (BSL1) sulfur-oxidizing bacteria. *T. thioparus* was grown in batch cultures of *Thiobacillus* medium DSMZ 36 (TM) which is composed of biological grade reagents including 0.1 g/L (NH₄)₂SO₄, 4 g/L K₂HPO₄, 4 g/L KH₂PO₄, 0.1 g/L MgSO₄·7H₂O, 0.1 g/L CaCl₂, 0.02 g/L FeCl₃·6H₂O, 0.02 g/L MnSO₄·H₂O, 10 g/L Na₂S₂O₃·5H₂O (Sigma-Aldrich and Fisher scientific) unless specified otherwise. Thiosulfate was used as primary energy sources for its metabolism and the phosphate salts in the medium conditioned the initial pH at 6.6 (Kelly et al., 2015). On the other hand, *S. novella* was also cultured in a modified *Thiobacillus* medium DSMZ 36 (TM1–6) supplemented with glucose (1.5 g/L, Fisher scientific) and yeast extract (0.3 and 5 g/L, Sigma-Aldrich) to favor its mixotrophic growth (Table 1), which considered the utilization of organic and inorganic substances (Leefeldt and Matin, 1980; Purnomo et al., 2019). Furthermore, *S. novella* was also grown in *S. novella* medium DSMZ 69 (SM) containing 10.6 g/L Na₂HPO₄·12H₂O, 1.5 g/L KH₂PO₄, 0.3 g/L NH₄Cl, 0.3 g/L yeast extract, 2 mL/L phenol red solution (0.1% w/v), 0.1 g/L MgSO₄·7H₂O, 5 g/L Na₂S₂O₃·5H₂O (Sigma-Aldrich and Fisher scientific), 5 mL/L trace element solution (MD-TMS, Cedarlane) as well as Trypticase soy broth medium DSMZ 535 (BM) comprised of 30 g/L trypticase soy broth (Fisher scientific).

Cultivation of these microbes was carried out in a rotatory shaking incubator (Biobase; BJPX-2102C) at 30°C and mixing speed of 150 RPM using 5% v/v inoculum and 500 mL Erlenmeyer flasks with 200 mL of working volume, unless stated otherwise. Before the experiments, all glassware was autoclaved at 121°C for 20 min and solutions were filtered with 0.2 μm Thermo Scientific Nalgene Rapid-Flow filter to maintain aseptic conditions. Optimal bacterial growth conditions for subsequent bio-oxidation

tests were studied by monitoring the pH, oxidation-reduction potential (ORP), dissolved oxygen (DO), optical density at 600 nm (OD_{600nm}), bacterial density (cells/mL) and thiosulfate consumption (mg/L). Both microorganisms were subjected to an adaptation process to the sulfidic ore for 3 months following the method of Astudillo and Acevedo (2008) and were used in the experiments. Nevertheless, the adaptation was fruitless over the considered adaptation time compared to the original strains. All experiments were carried out in duplicates throughout this investigation.

Bio-oxidation experiments

In the first instance, elemental sulfur (S⁰) was used in bio-oxidation tests to evaluate the potential of both microorganisms to oxidize solid matrices. A concentration of 5,000 mg/L S⁰ was initially employed as a sulfur material in bio-oxidation tests using a two-step methodology unless stated otherwise. A two-step bio-oxidation technique involves tests at which bacterial inoculum (5% w/v) of an existing culture is originally incubated in a fresh medium and allows to propagate using the medium components, e.g., thiosulfate, as an initial energy source to achieve an optimal growth. After the exponential phase of the bacterial growth (2–3 days) was accomplished, the sulfur material to be oxidized was introduced into the reactor, commencing the two-step reaction. Tests were conducted in a rotatory shaking incubator at 30°C and 150 RPM using 500 mL Erlenmeyer flasks with a working volume of 200 mL for a total experimental time of 10 days. pH, ORP, DO, and bacterial density were monitored throughout the tests. Control tests were aseptically carried out under identical testing conditions using the same bacterial growth medium with no microorganisms added.

Subsequently, the oxidative capacities of these neutrophilic microorganisms were tested with a refractory sulfidic ore following a similar experimental procedure used for S⁰, as specified in Table 2. Thus, once the exponential phase of the bacterial growth was successfully reached, a sulfidic ores sample at a solid to liquid ratio of 10–100 g/L was added into the reactor. Bio-oxidation tests with sulfidic ores were mainly performed in 500 mL Erlenmeyer flasks at the ideal conditions for bacterial growth mentioned in the previous section in a temperature-controlled incubator for 10 days of reaction time. Thymol (1% m/v) wash was used to ensure there was no other culture already present in the sulfidic ore before starting the tests (de Carvalho et al., 2019). 2 mL aliquots were withdrawn to monitor bacterial growth, pH, ORP and DO. Following tests in 500 mL reactors, bio-oxidation reactions with the refractory sulfidic ore were scaled up in a 1 L jacketed reactor (Chemglass) equipped with overhead mixing at 150 RPM as shown in Figure 1. The temperature was adjusted to 30°C, and the air was sparged at 0.1 L/min to maintain DO concentration throughout the test. A volume of 950 mL of fresh culture medium was added to the reactor, followed by 5% w/v inoculum of active bacterial culture, which was grown 2–3 days before starting the test with the ore. After 10 days of contact time, the reactor test was ended, and a liquid aliquot was taken for further analysis. The pulp was pressure-filtered, separating solid residue from the liquid phase and the solid residue was washed with deionized water and filtered one more time.

TABLE 2 Experimental conditions bio-oxidation of refractory sulfidic ores.

Factors	Shake flask	Bioreactor
Solid to liquid ratio (g/L)	10–100	10
Inoculum (% w/v)	5	5
Initial pH	6.6–7.5	6.6
Mixing speed (RPM)	150	150
Type of motion	Orbital	Stirring
Time of reaction (days)	5–30	10

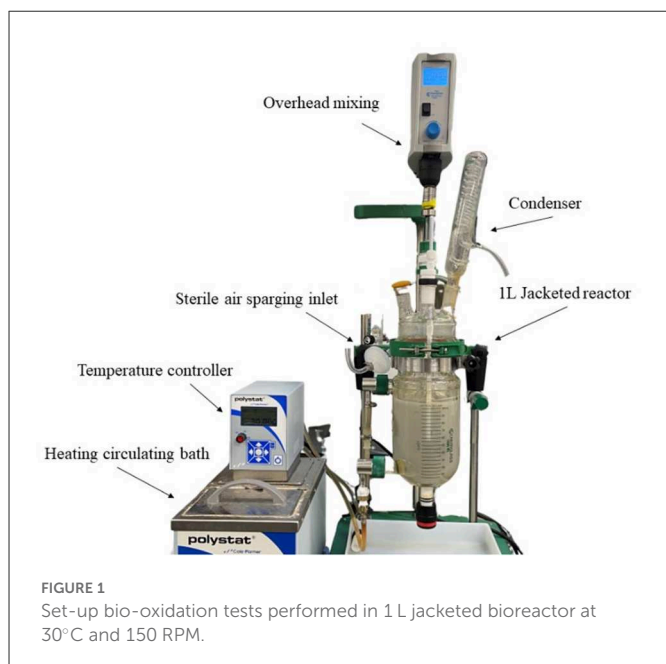


FIGURE 1 Set-up bio-oxidation tests performed in 1 L jacketed bioreactor at 30°C and 150 RPM.

Analytical techniques

The mineralogy of the sulfidic ore was determined by X-ray diffraction analysis (XRD; Philips, Netherlands) and the morphology of solid surfaces was characterized with scanning electron microscopy (SEM; Bruker AXS Microanalysis GmbH, Germany). The concentration of elements in the sulfidic ores was determined with coupled plasma-optical emission spectrometry (ICP-OES; PerkinElmer ELAN 9000/NexION).

Cell growth was monitored with ultraviolet–visible spectroscopy (UV-VIS; Thermo Scientific GENESYS 10S) contrasted against aseptic culture medium at 600 nm and manual cell counting employing a Neubauer counting chamber and a Rebel microscope (ECHO) at 40–100× magnification following the method of Abcam (2022) throughout the tests as shown in Supplementary Figure S1. Liquid samples were filtrated with 0.45 μm sterile syringe filter before further analysis. pH and oxidation-reduction potential (ORP) were monitored utilizing a saturated Ag/AgCl electrode on a multichannel pH/ORP probe (VWR; Symphony H30PCO). Dissolved oxygen (DO) was obtained using an optical DO sensor connected to a multimeter (METTLER TOLEDO, Fisher Scientific). Thiosulfate concentration was determined with iodometric titration using the method of Arima et al. (2004) and sulfate concentration was measured with ion chromatography (IC; Methrohm 930 Compact IC Flex).

After S⁰ bio-oxidation, S⁰ was washed with DI water and dried in a vacuum-sealed oven below 40°C (Liu et al., 2013). The oxidation rate in S⁰ bio-oxidation was estimated by considering the concentration of initial elemental sulfur and the concentration of elemental sulfur in the final residue. Hence, sulfur oxidation was therefore calculated as shown in Equation 3:

$$S_{S^0-S_{oxidation}} = \frac{(m_{S^0-S_{initial}} - m_{S^0-S_{residue}}) \times 100}{m_{S^0-S_{initial}}} \quad (3)$$

where $m_{S^0-S_{initial}}$ represent the mass (g) of S⁰ initially and $m_{S^0-S_{residue}}$ stands for mass (g) S⁰-S in the residue.

Moreover, sulfide oxidation rate in sulfidic ore was determined using carbon/sulfur analyzer (Eltra CS 200) (Ofori-Sarpong et al., 2011; Kaksonen et al., 2014a). After finishing the tests, the oxidized sample was separated and dried in a vacuum-sealed oven below 40°C. The treated sample was washed with toluene in a ratio of 1 g: 50 mL at 60°C for 60 min to remove any S⁰ formed during bio-oxidation and dried under identical conditions (Marzoughi et al., 2022). Subsequently, the oxidized sample was washed with 3M hydrochloric acid (HCl) at 65°C for 90 min to remove sulfate (SO₄²⁻) (Ofori-Sarpong et al., 2011). The washed residues were dried and further analyzed with carbon/sulfur analyzer to determine the sulfide oxidation rate. The oxidation rate was calculated based on the total sulfide concentration in the residue as displayed in Equation 4 (Kaksonen et al., 2014a):

$$S_{sulfide-S_{oxidation}} = \frac{(m_{sulfide-S_{ore}} - m_{sulfide-S_{residue}}) \times 100}{m_{sulfide-S_{ore}}} \quad (4)$$

wherein $m_{sulfide-S_{ore}}$ stands for the mass (g) of sulfide-S in the original ore and $m_{sulfide-S_{residue}}$ represent mass (g) sulfide-S in the residue after washing with toluene and HCl.

Results and discussion

Sample characterization

The composition of the sample is presented in Table 3. The low-grade refractory sulfidic ore sample contained 5.7% w/w total sulfur and 1.7 ppm Au with a minor content of heavy metals. Moreover, Figure 2A displays the XRD pattern of the refractory sulfidic ores used in bio-oxidation. The main phases in the ore were quartz, muscovite, greigite, spangolite, and pyrite. Further, Figure 2B shows the particle size of 80% passing (P80) which was determined to be 55 μm.

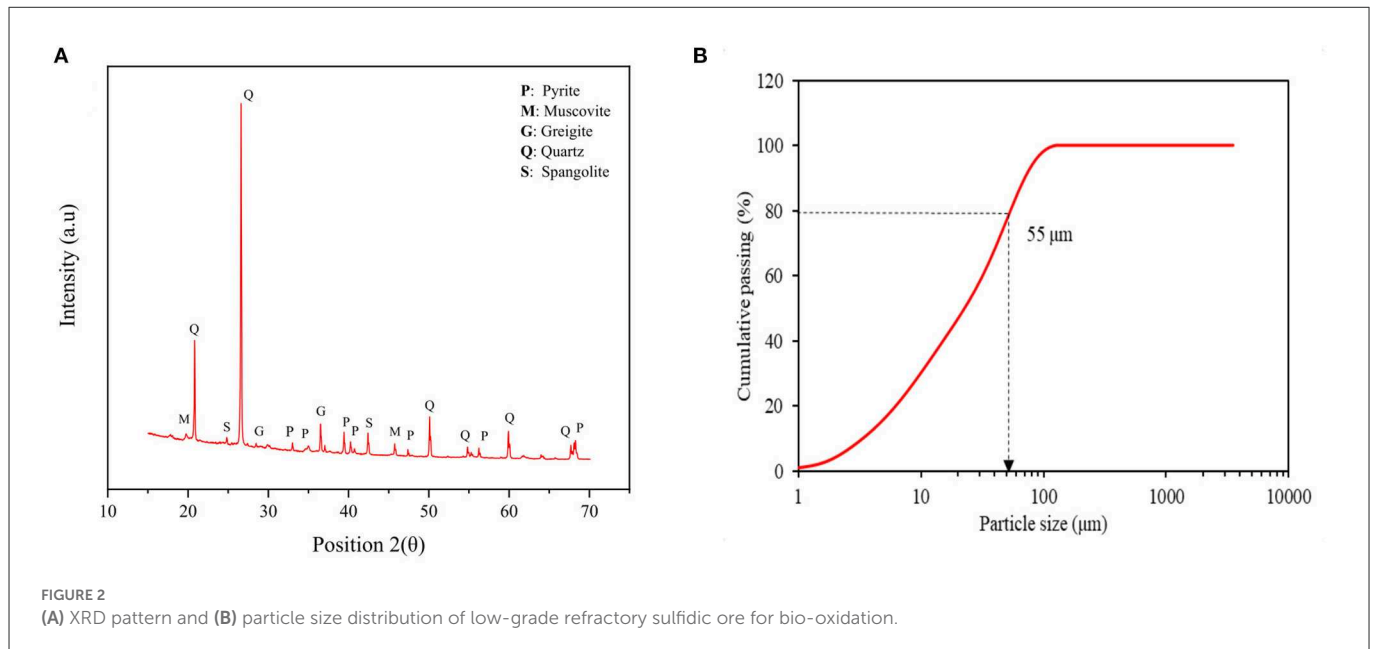
Effect of medium composition on bacterial growth

The bacterial growth of both neutrophilic bacteria was evaluated in response to changes in the concentration of certain ingredients in the culture media. Figures 3, 4 show the changes in bacterial growth as well as the fluctuations in pH, ORP (mv), and measured thiosulfate concentration (mg/L) over time for *T. thio-parus* and *S. novella*, respectively.

In the case of *T. thio-parus* (Figure 3), a significant impact was produced by the change in the initial concentration of sodium

TABLE 3 Composition of metals in low grade refractory sulfidic ore.

Element	Ag	Au	Al	Co	Cu	Fe	Mg	Ni	S	V
Concentration (ppm)	6.3	1.7	54,300	76	661	50,700	2,820	18	5.7%	80



thiosulfate in the culture medium, influencing the main parameters measured during its growth. From Figures 3A, B, the exponential phase of the bacterial growth was achieved after 3 days in all the experimental conditions, abruptly increasing optical density and bacterial density, correspondently. Optical density reflects the bacterial growth based on the turbidity of the bacterial solution (Widdel, 2010), which is normally determined at a wavelength of 600 nm in the case of the selected bacteria (Toth et al., 2015; Peighami et al., 2022). Thus, an increase in OD₆₀₀ indicates that the bacterial population increases over time which is reflected in more turbid solutions compared to the original solution at day 0 in Figure 3. An increase in sodium thiosulfate concentration from 2 to 10 g/L led to a substantial rise of 2–3 folds in OD₆₀₀ and bacterial density as thiosulfate is utilized as the principal energy source for the bacteria, favoring its metabolism at higher concentration in the range 2–10 g/L.

The more significant growth using 10 g/L sodium thiosulfate could be attributed to its metabolic characteristic since *T. thioparus* is an obligately chemolithotrophic bacteria that obtains its energy mainly from the oxidation of reduced sulfur compounds such as thiosulfate presented in the culture medium (Kelly et al., 2015). Reduced sulfur species are used as electron donors and oxygen is utilized as an electron acceptor to generate an electron flow in the cell membranes required to satisfy the bacterial metabolism through phosphorylation reactions (Kelly et al., 2015; Lee et al., 2021). Therefore, an increase in the concentration of sodium thiosulfate in the range 2–10 g/L seems to have helped to enhance the metabolism of *T. thioparus*, which resulted in an augment in OD₆₀₀ > 0.6 with a bacterial density of 1.2×10^6 cells/mL.

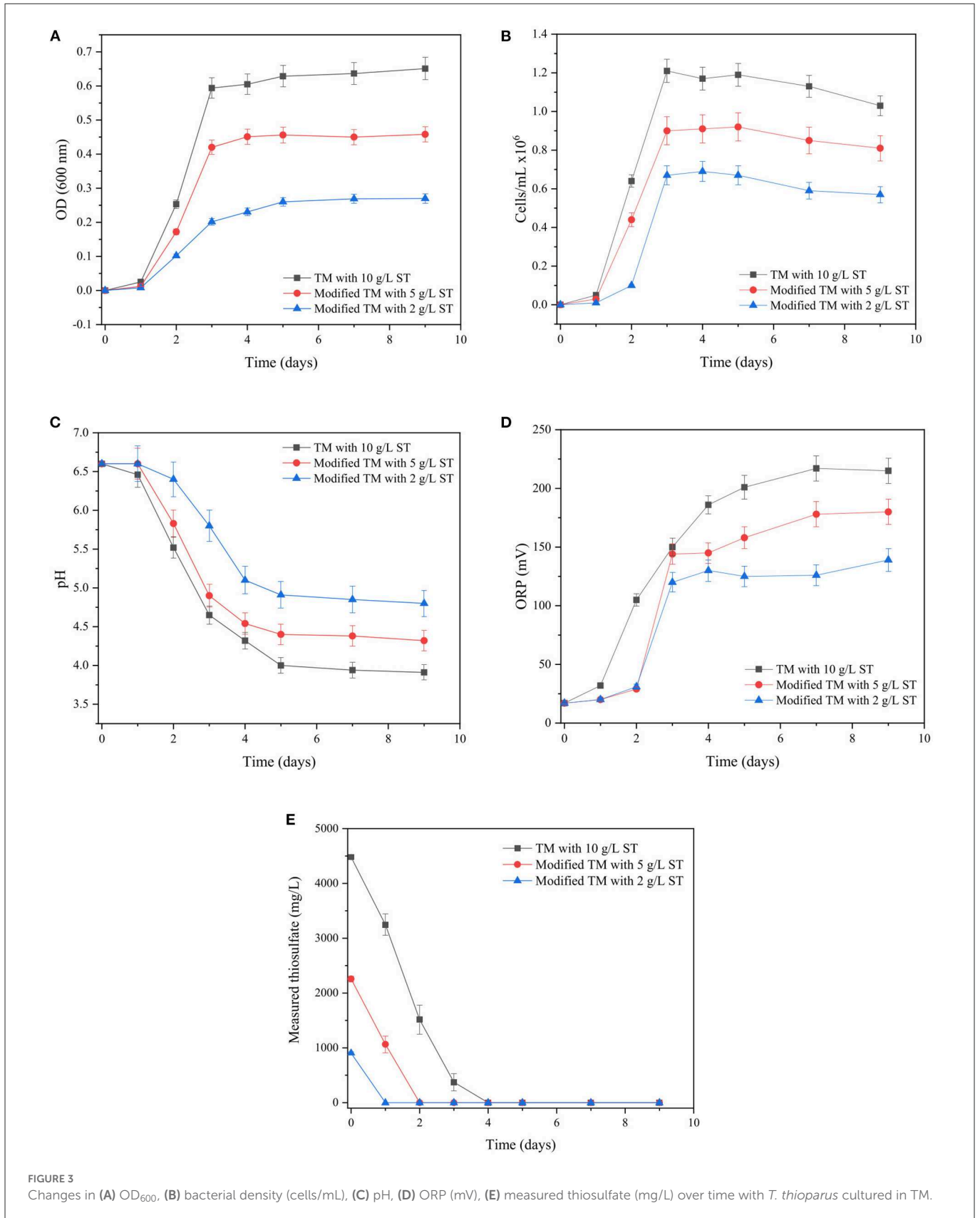
A concentration higher than 10 g/L sodium thiosulfate was also employed to enhance the bacterial growth of *T.*

thioparus. Nevertheless, a concentration of 20 g/L sodium thiosulfate (Supplementary Figure S2) had a negative impact, which seems to be related to a possible salinity stress generating a opposite effect on bacterial growth. Higher salinity in the medium affects the water balance of bacterial membranes due to its osmotic properties, which depends on the class of bacteria and environmental conditions (Boziaris et al., 2007; Kumar et al., 2021). Keller (1969) noted that the growth of *T. thioparus* ATCC 8158 was entirely inhibited at 0.3 M NaCl due to low tolerance to high saline environments.

Furthermore, Figures 3C, D show that a higher concentration of sodium thiosulfate (2–10 g/L) caused a more abrupt decrease in the solution pH and an increase in ORP during the evaluated period. A drop in pH was observed from initial pH 6.6 to pH 4.0–5.0 when the exponential phase of the growth was reached. ORP increased during the exponential phase and then remained in the range 130–230 mV depending on the initial sodium thiosulfate provided.

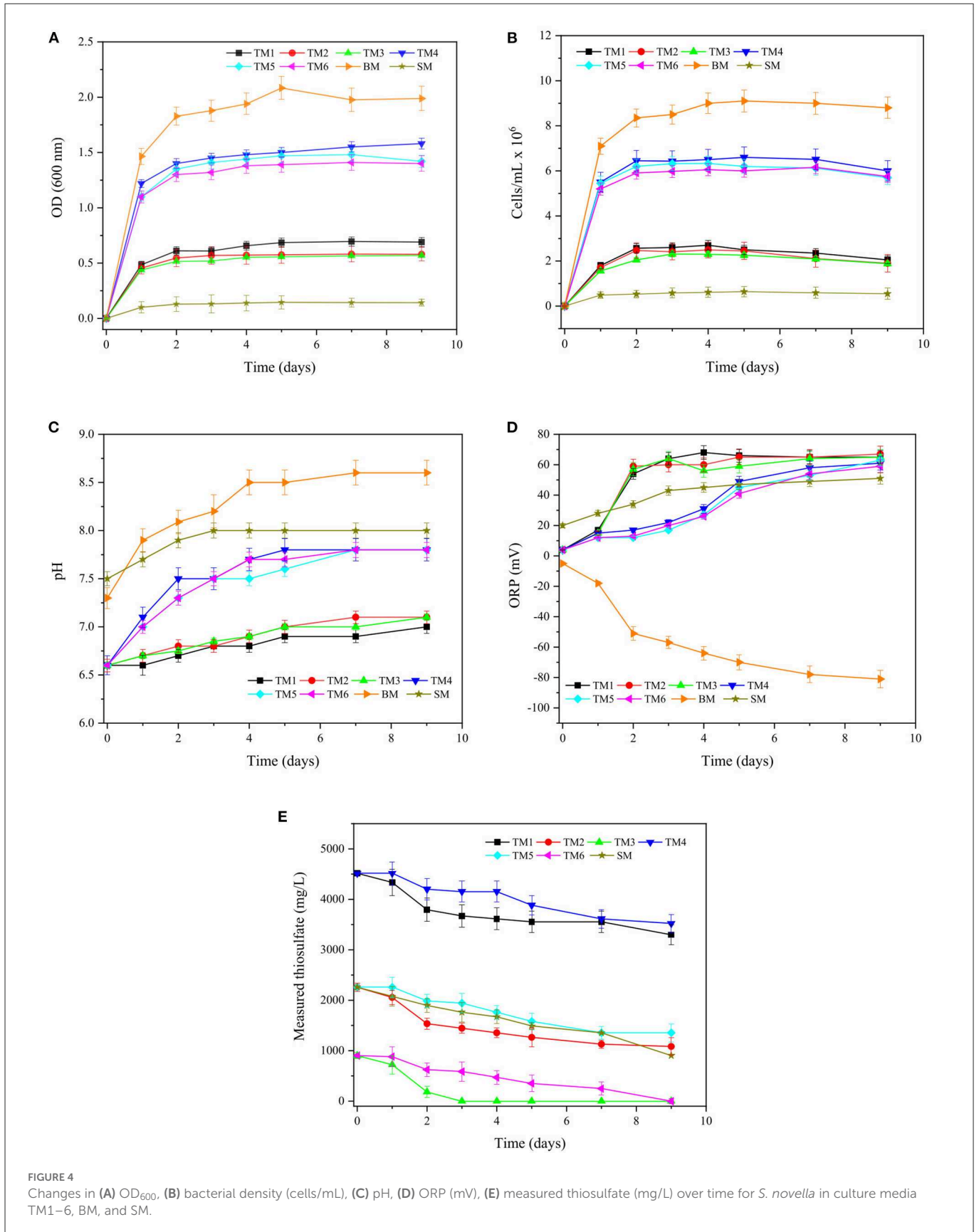
The decrease in pH and increase in ORP could be caused by the metabolism of thiosulfate during the bacterial growth and the release of protons into the medium by *T. thioparus* (Kelly, 2006; Tang et al., 2009). Thiosulfate was undetectable at the end of the bacterial growth in all the culture media as shown in Figure 3E. Control tests kept a near-neutral pH and ORP < 50 mV with insignificant changes in thiosulfate concentration compared with the test (data not shown), demonstrating that the *T. thioparus* was responsible for the changes observed during the evaluated interval. A concentration of 10 g/L sodium thiosulfate was preferred during the evaluation of its oxidative capacities of solid matrices due to a more robust growth was obtained under these conditions.

Figure 4 displays the bacterial growth of *S. novella* cultured in modified TM (Table 1), BM, and SM. *S. novella* is a facultative



chemolithotrophic sulfur-oxidizing bacterium that can thrive using both organic and inorganic reagents to obtain energy required for its metabolism (Perez and Matin, 1980; Kelly and Wood, 2000). Media

TM1-6 and SM favored a mixotrophic growth using organic and inorganic compounds, while medium BM supported a heterotrophic growth based on the utilization of only organic substrates.



The exponential phase of the bacterial growth was reached after 1–2 days of incubation in all the culture media as shown in Figure 4A, which resulted in an abrupt increase in OD in all media and thus more

turbid solutions compared to the respective original culture medium at day 0 owing to a more significant number of microorganisms. In media TM1–6, the bacterial growth was increased almost 3 folds as

the concentration of yeast extract was augmented from 0.3 to 5 g/L. Organic reagents such as yeast extract, are used by *S. novella* as a source of carbon required for its growth (Kelly et al., 2000). In particular, yeast extract is a nourishing nutrient with a high diversity of proteins, amino acids, and minerals (Pasupuleti and Demain, 2010). Thus, adding a higher concentration of yeast extract in the culture medium could stimulate bacterial growth notably in terms of OD₆₀₀ and bacterial density as shown in Figures 4A, B, respectively.

However, even though the bacterial growth of *S. novella* resulted in higher OD in culture media with 5 g/L yeast extract (TM4–6), the utilization of thiosulfate was slower compared to cultures in TM1–3 as illustrated in Figure 4E. The ability of *S. novella* to oxidize reduced sulfur species could be affected by an excess of yeast extract causing an inhibition of substrate utilization, as the microorganism might prefer other substrates for its metabolism such as organic reagents instead of thiosulfate (Leefeldt and Matin, 1980). When organic reagents were not provided in TM, the growth of *S. novella* was completely inhibited (data not shown) due to the requirement of organic reagents (Leefeldt and Matin, 1980; Kelly and Wood, 2000). Contrasting *T. thioparus*, the variation in sodium thiosulfate concentration in the range of 2–10 g/L in the modified TM1–6 was not an influential parameter for *S. novella*, producing a slight change in OD₆₀₀ and bacterial density (Figures 4A, B).

Medium SM was also evaluated under similar conditions; nonetheless, the bacterial growth in OD₆₀₀ and bacterial density was 4–6 times lower than TM1–6, which is reflected in the bacterial density (cells/mL). The difference between TM1–6 and SM seems to have been attributed to the addition of another exogenous organic matter in TM1–6, e.g., glucose, which has been noted to support bacterial metabolism and cell growth of *S. novella* (Rittenberg, 1969; Leefeldt and Matin, 1980; Kelly and Wood, 2000). The bacterial growth was also investigated in a pure organic medium (BM) containing trypticase soy broth, which is a substrate mainly composed of tryptone, a rich source of amino acid (Pasupuleti and Demain, 2010). This culture medium produced a noticeably more significant growth than TM1–6 and SM, which resulted in an OD₆₀₀ > 2. However, the behavior of the bacteria under heterotrophic conditions could be different to mixotrophic growth due to dissimilar enzyme production as demonstrated by Aleem (1965) and Leefeldt and Matin (1980).

As shown in Figures 4C, D, pH and ORP generally increased during exponential phase and then stayed in the range 7.0–8.5 and 20–60 mV in most of the cases. Culture media containing yeast extract and tryptone showed an increase in pH due to the buffer capacity of these reagents, which seems to be associated with the decomposition of organic components and formation of alkaline products generated during bacterial growth (Losen et al., 2004). The increase in ORP in most of the culture media could be attributed to the metabolism of thiosulfate, as explained in the previous section. Additionally, it is worth mentioning that *S. novella* consumed up to 1,000 mg/L thiosulfate in all the experimental conditions, which was ca. 4 times lower than *T. thioparus*, causing a minor change in pH and ORP. Therefore, *T. thioparus* might have a higher potential to oxidize solid matrices than *S. novella*. Besides, the absence of thiosulfate in BM composition (not shown in Figure 4E) could have influenced

the decreasing trend in ORP, reaching ca –80 mV at the end of the incubation.

Although all the culture media were tested for sulfide oxidation, TM3 was preferred in future experimentation with solid matrices since *S. novella* metabolized most of the initial thiosulfate presented in the solution after the exponential compared to the other cases, which could lead to increase the possibilities of oxidizing the sulfidic ore.

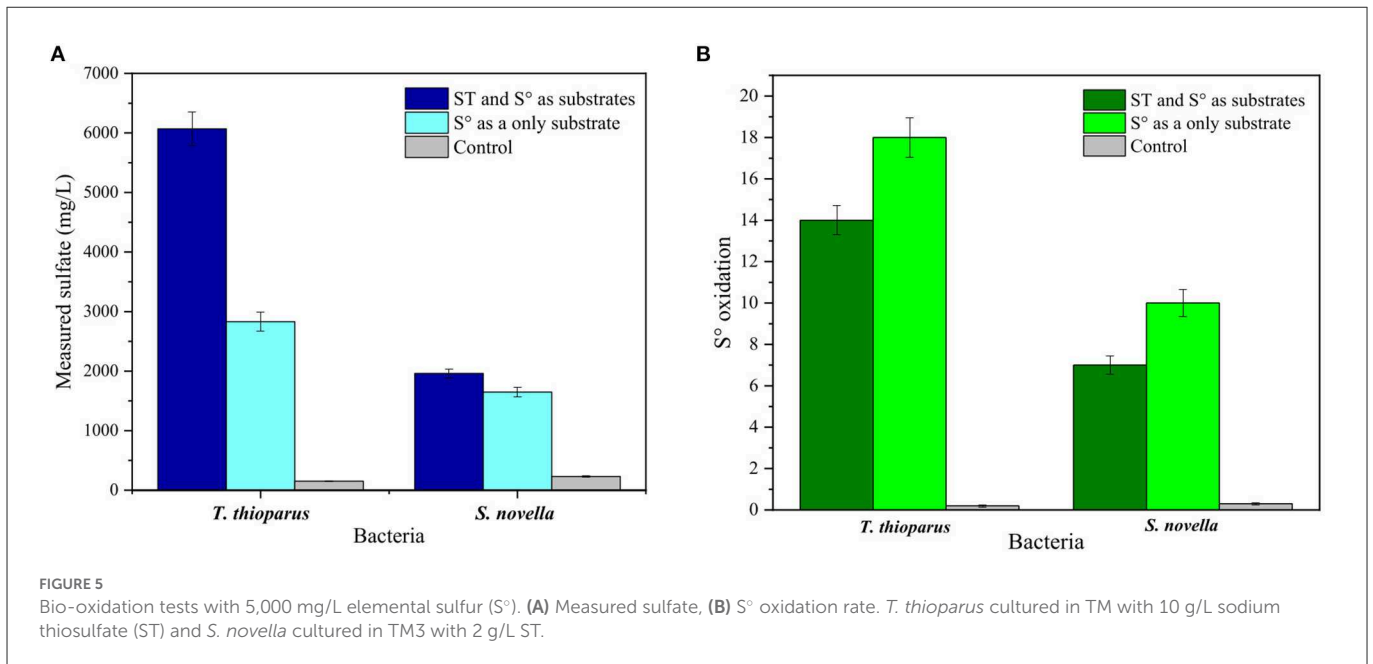
Bio-oxidation tests with elemental sulfur

A preliminary assessment was performed with elemental sulfur (S⁰) to explore the oxidative capacities of *T. thioparus* and *S. novella* on solid matrices. Although there is no extensive research on S⁰ oxidation with these specific microorganisms, neutrophilic bacteria such as *T. thioparus* and *S. novella* have been commonly encountered in soils, showing a more notorious activity in soils containing S⁰ due to their bacterial metabolism (Deluca et al., 1989; Chapman, 1990; Germida and Janzen, 1993; Pourbabaee et al., 2020).

Bio-oxidation tests with S⁰ were used as a baseline for subsequent bio-oxidation tests with sulfidic ores. Two different approaches were implemented to confirm the ability of both microorganisms to oxidize S⁰. On the one hand, the bacteria were first grown with thiosulfate as an initial energy source until the exponential phase was achieved, and subsequently, 5,000 mg/L S⁰ was added into the reactor. On the other hand, the bacteria were directly grown with S⁰ (5,000 mg/L) as sole energy source material with no addition of thiosulfate in the medium. Figure 5 shows a comparison between both strategies along with the control tests for *T. thioparus* and *S. novella*.

Thiobacillus thioparus showed more significant potential than *S. novella* in oxidizing S⁰ using both approaches. When S⁰ was used as the only substrate, 2,830 mg/L sulfate was measured in the presence of *T. thioparus*, which was almost 2 folds more sulfate compared to *S. novella* under similar conditions. A total S⁰ oxidation rate of ca. 18% was oxidized by *T. thioparus* after 10 days of reaction time, while 10% of total S⁰ were oxidized by *S. novella* (Figure 5B). During the tests with S⁰ as the only energy source, *T. thioparus* increased solution acidity to pH 4, while *S. novella* maintained a near-neutral pH (data not shown). Chapman (1990) found that the addition of S⁰ increased *T. thioparus* bacterial density, reaching 10⁵ cells/g soil and more acid pH solution, and Lindemann et al. (1991) showed similar results with the same microorganism. In another study, Boretska and Bellenberg (2013) pointed out that S⁰ stimulated *T. thioparus* to produce different extracellular polymeric substances (EPS), improving bacterial attachment in solid substrates. On the other hand, Pourbabaee et al. (2020) reported a closely related strain to *S. novella* that produced ca. 12–15% S oxidation with 0.3-unit pH variation over 2 weeks, which agreed with the results shown in Figure 5B.

By using thiosulfate as an initial substrate for bacterial growth before the addition of S⁰, *T. thioparus* formed ca. 6,000 ppm (Figure 5A). However, the oxidation rate of S⁰ was 14%, which was slightly lower than the previous approach. The decrease in oxidation could be produced by the presence of thiosulfate, which also served as a source of energy for the bacterium (Kelly et al., 2015). *S. novella*



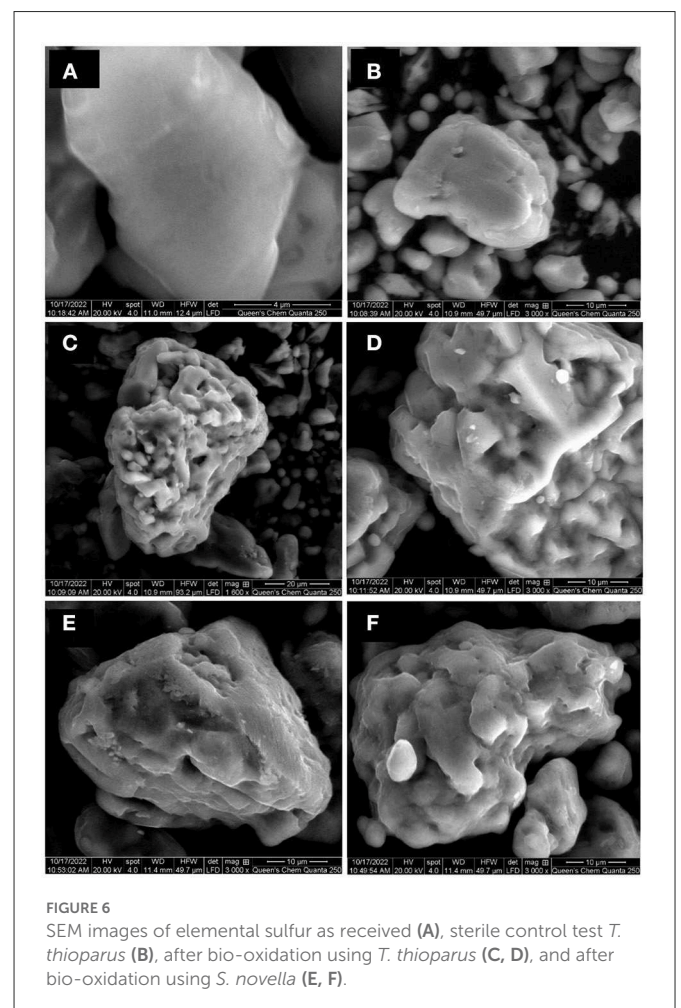
cultured with 2 g/L sodium thiosulfate in TM3 showed that ca. 7% of the initial S° was oxidized in the medium. Blank tests showed a negligible formation of sulfate in the range 150–250 mg/L, which could be caused by the presence of traces of sulfate salts in the culture medium and minor chemical oxidation. The oxidation of S° produced by abiotic factors has been noticed to be limited in soils compared to biological reactions (Germida and Janzen, 1993), which agreed with the results displayed in Figure 5.

From Figure 5, it is clear that both microorganisms are able to oxidize solid matrices, achieving a maximum of 18% oxidation with *T. thioparus* over 10 days. Although there was no complete oxidation of S° , the sulfur content of the refractory gold ore used in this study is 5.7%, which is lower than the amount of S° utilized in this section. Thus, this opens an option to achieve higher oxidation when working with the refractory sulfidic ore compared to S° .

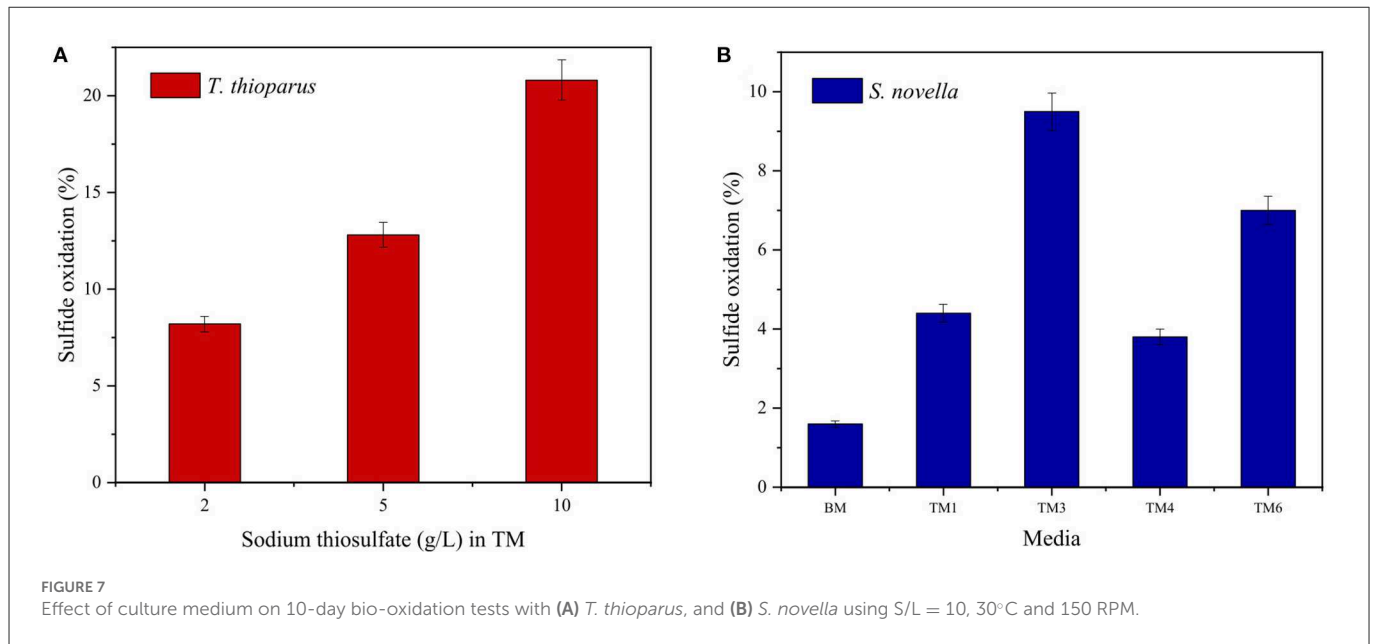
The surface morphology of S° before and after bio-oxidation was evaluated with SEM images (Figure 6). The surface of the S° before bio-oxidation was characterized by a smooth texture, and the S° surface obtained after sterile control showed subtle surface alterations compared to the as-received S° sample (Figures 6A, B). Liu et al. (2013) also confirmed that the S° surface in control tests remained almost unchanged and that the roughness of the S° surface was produced by bacterial action.

Figures 6C–F show that the S° surface after contact with *T. thioparus* and *S. novella* became rougher compared to as received S° (Figure 6A). It was observed that *T. thioparus* led to more severe corrosion than *S. novella*, with more alteration and pits. The changes on S° surface were similar to the alteration produced by common acidophilic microorganisms (Briand et al., 1999). The bacterial action was generally irregular on the S° surface, causing non-uniform distortions (Espejo and Romero, 1987), which was less noticeable in the bio-oxidation test with *S. novella* due to the lower oxidation rate.

The more remarkable bacterial alteration produced by *T. thioparus* seems to be related to the higher sulfate production compared to *S. novella*. Briand et al. (1999) also noted more corroded surfaces led to a higher sulfate generation due to S° utilization in



average 10–14 mmol/day. However, the sulfur consumption rate with neutrophilic bacteria was observed to be lower, which was reflected



in the less corroded surfaces compared to acidophilic bacteria such as *T. thiooxidans* and *A. ferrooxidans*.

Bio-oxidation tests with refractory sulfidic ore

Effect of culture medium on bio-oxidation tests

After confirming that the microorganisms were capable of oxidizing S° , the bio-oxidation of refractory sulfidic ore with both neutrophilic bacteria was further explored using selected culture media. Figure 7 displays 10-day bio-oxidation tests of refractory sulfidic ores. Different concentrations of sodium thiosulfate in the culture media were tested to promote the biological oxidation of sulfide in the sulfidic ore (Mubarak et al., 2017).

From Figure 7, *T. thioparus* displayed a higher potential in bio-oxidation of sulfidic ores than *S. novella* showing the same trend observed with S° with ca. 50% higher oxidation rate. *T. thioparus* increased the oxidation rate from 8.2 to 20.8% when the initial concentration of sodium thiosulfate in TM augmented from 2 to 10 g/L. Contrary to Mubarak et al. (2017), a higher sodium thiosulfate concentration in the culture medium led to a higher oxidation rate. The initial bacterial growth of *T. thioparus* mainly depended on the initial concentration of sodium thiosulfate, which was almost consumed during the exponential phase growth. Therefore, a concentration of 10 g/L resulted in a bacterial density of 1.2×10^6 cells/mL, which was 2 times higher compared to TM with 2 g/L sodium thiosulfate. Sajjad et al. (2019) pointed out that a greater bacterial density might result in a higher bio-oxidation rate, which agreed with the results found in Figure 7A. Control tests in TM with varied concentration sodium thiosulfate resulted in negligible oxidation <1% (data not shown).

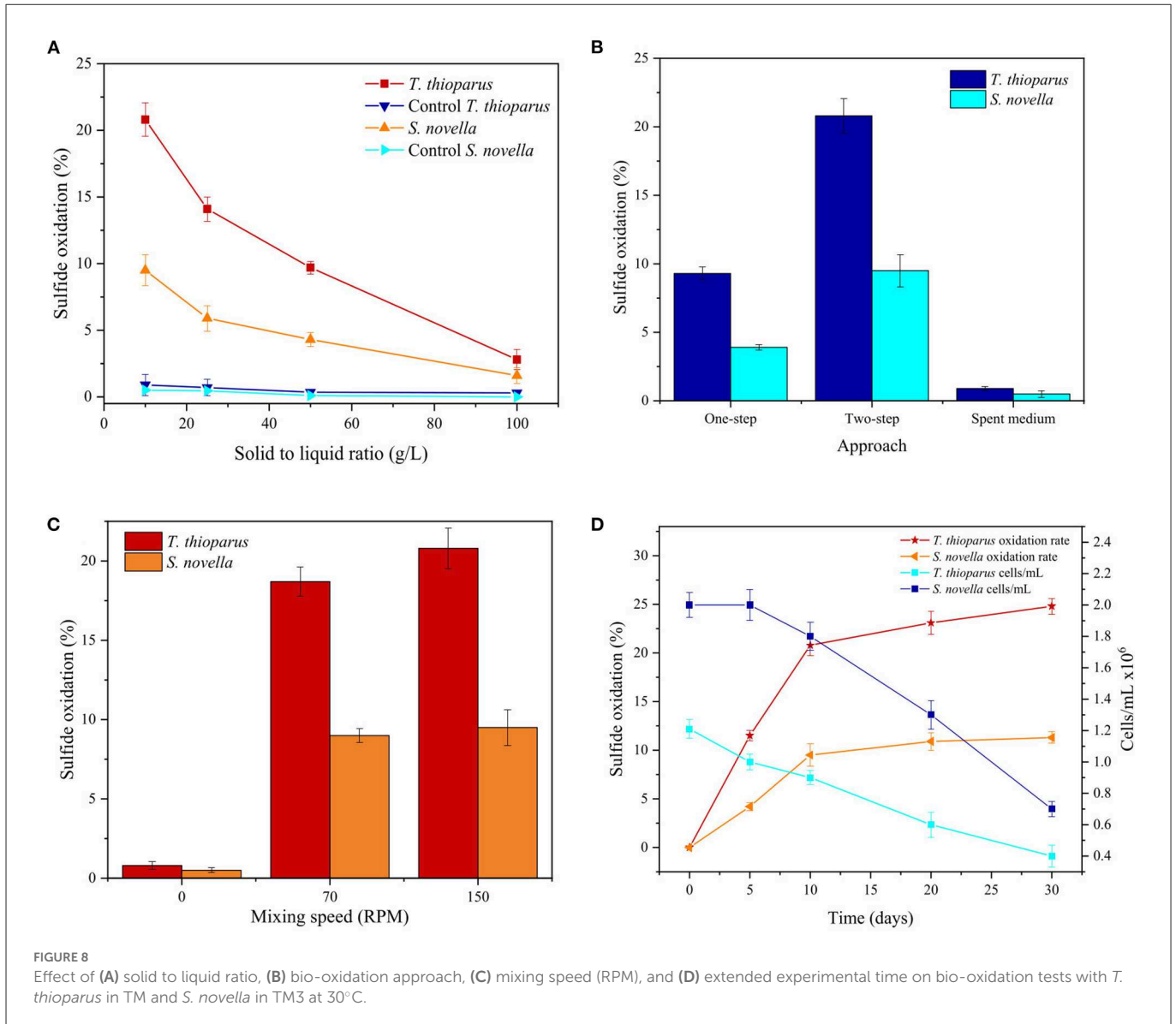
Contrary to *T. thioparus*, 2 g/L sodium thiosulfate favored the oxidation of the sulfidic ore with *S. novella* (Figure 7B), which resulted in 9.5 (TM3) and 7.9% (TM6) as suggested by Mubarak et al. (2017). BM, a nutrient-rich medium composed of trypticase soy

broth, had a negligible sulfide oxidation rate despite the fact that this medium generated the highest OD and bacterial density compared to other culture media. An excess of nutrients in the culture medium could inhibit substrate utilization affecting the oxidative capacities of the bacteria and enzyme production. In a medium with an excess of nutrients, it might be possible that microorganisms have enough resources in terms of thiosulfate and organic reagents to thrive in the medium, decreasing the probability of oxidizing the ore. (Leefeldt and Matin, 1980; Mubarak et al., 2017). All the control tests also showed insignificant sulfur oxidation compared to tests with *S. novella* (data not shown).

Effect of modification of experimental parameters on bio-oxidation tests

Experimental parameters such as the solid to liquid ratio (g/L), bio-oxidation approach, mixing speed (RPM), and time of reaction (days) were further investigated, and the results are summarized in Figure 8. By augmenting the solid-to-liquid ratio from 10 to 100, sulfide oxidation using *T. thioparus* decreased from 20.8 to 3.8% over 10 days. *S. novella* showed a similar trend, decreasing oxidation rate from 9.5 to 1.6%, as shown in Figure 8A. The effect of higher solid-to-liquid ratios in bacterial performance has been addressed to possible poor mass transfer and heavier environmental stress due to higher concentrations of solids and heavy metals presented in the tested samples, affecting the bacterial performance and resulting in lower oxidation (Azizitorghabeh et al., 2022).

Furthermore, different approaches were explored for bio-oxidation including one-step, two-step and spent medium. Two-step bio-oxidation was used in most of the tests carried out in this investigation. However, there are other specific approaches to conducting tests with microorganisms, encompassing one-step and spent medium techniques (Faraji et al., 2018). Figure 8B compares each method using both bacteria. It was noted that one-step tests produced approximately half the sulfide oxidation compared to two-step tests. The decrease in oxidation rate could be caused by the lower concentration of microorganisms at the beginning of one-step



test contracting with two-step test with an initial population $>10^6$ cells/ mL (Sajjad et al., 2019). In the spent medium technique, tests were carried out only with cell-free metabolites generated during the bacterial growth, which might include extracellular polymeric substances (EPS) of both bacteria (Gehrke et al., 1998). This approach was also more ineffective than the two-step methodology as sulfide oxidation was similar to the control tests, implying that microorganisms form part of the oxidation mechanism as reported in standard bio-oxidation using acidophilic bacteria (Azizitorghabeh et al., 2022).

Bacterial attachment to the sulfidic ore surface is essential in the bio-oxidation mechanism with microorganisms. A proper bacterial attachment influences biofilm formation, which is a protective barrier for cells during bio-oxidation (Boretska and Bellenberg, 2013). Thus, it is likely that bacterial attachment also has a predominant role in the case of neutrophilic bacteria. Bio-oxidation tests using <150 RPM were proposed to improve the adherence of cells to the refractory sulfidic ore, and the results are displayed in Figure 8C. Nevertheless, the results revealed that a decrease in mixing speed was an ineffective

parameter in boosting biological oxidation with *T. thioparus* and *S. novella*. The reduction of mixing speed could lead to bacterial sedimentation into the bottom of the reactor affecting the circulation of microorganisms and proper aeration of the culture medium, thus preventing an adequate mixture with the medium and substrates (Bates et al., 2016).

Moreover, experimental time is also an essential parameter in increasing sulfide oxidation biologically (Sajjad et al., 2019). Figure 8D shows the effect of time in bio-oxidation and bacterial population in 30 days. The results revealed that sulfide oxidation increased markedly within the initial 10 days, reaching 20.8 and 9.5% sulfide oxidation for *T. thioparus* and *S. novella*, respectively. Subsequently, sulfide oxidation rate was stalled resulting ca. 24.8 and 11.3% sulfide oxidation correspondingly within 30 days reaction. It was also noted that the bacterial density of both microorganisms decreased $>50\%$ with 30 days which might also affected the oxidation of sulfide (Sajjad et al., 2019). The deceleration in sulfide oxidation rate could also be caused by a depletion of nutrients and environmental conditions e.g., decrease in pH during more extended

TABLE 4 Comparison bio-oxidation tests in bioreactor and shake flasks^a.

Condition	Factors Type of reactor	<i>T. thioparus</i>		<i>S. novella</i>	
		Bioreactor	Shake flasks	Bioreactor	Shake flask
Initial	Bacterial density (cells/mL × 10 ⁶)	1.4	1.2	2.4	2
	pH medium	6.6	6.6	6.6	6.6
	pH after exponential phase	4.2	4.6	6.2	6.6
	ORP (mV)	184	150	97	57
Final	Bacterial density (cells/mL × 10 ⁶)	0.8	0.9	2.1	1.8
	pH	3.7	4	7	7
	ORP (mV)	260	210	117	73
	Sulfide oxidation (%)	27.2	20.8	14	9.5
	Control sulfide oxidation (%)	1.0	0.9	0.9	0.5

^aTests using 1 g/L solid/liquid ratio, 5% v/v inoculum, 150 RPM, and 10 days reaction time.

periods influencing bacterial population (Li et al., 2016; Bulaev, 2020) as well as precipitation of iron phases on the surface of the sulfidic ore over time (Ofori-Sarpong et al., 2011).

Optimization and scale-up of reactions

Following bio-oxidation conducted in shake flasks, reactions were scaled up in a 1 L bioreactor, and Table 4 compares both type of experiments using both microorganisms. By using the bioreactor, sulfide oxidation with *T. thioparus* showed an increase from 20.8 to 27.2% with 10 days reaction time. Moreover, *S. novella* followed a similar tendency by augmenting sulfide oxidation from 9.5 to 14%, as displayed in Table 4. The bacterial density of both cultures also displayed an increase in the population of ca. 20% as well as more fluctuation in pH and ORP compared to shake flask tests (Table 4). Control tests in the bioreactor resulted in minor oxidation with negligible changes in measured parameters compared to shake flask control tests, confirming that the enhancement observed was biologically caused. The improvement in bacterial growth and sulfide oxidation could be attributed to the agitation mode in the bioreactor, which may have enhanced the fluid dynamics of the system favoring the heat and mass transfer in the culture as well as oxygen and nutrient supply availability due to better homogenization of the culture solution (Büchs, 2001; Eppendorf, 2018). Although the bioreactor promoted greater oxidation, acidophilic bacteria can still provide more effective oxidation. However, some strategies to increase oxidation could optimize a future process with neutrophilic bacteria, including replenishing the culture medium, simulating a continuous operation (Do et al., 2022), and incorporating a reactor equipped with multiple online sensors for real-time process control, encompassing nutrients and pH over time (Eppendorf, 2018).

Regarding the bacterial action, the oxidation mechanism of reduced sulfur species varies depending on the physiological characteristics of each bacterium, which can oxidize various reduced sulfur compounds with varied kinetics. Generally, sulfur-oxidizing bacteria obtain energy from the oxidation of reduced sulfur compounds, using oxygen as electron donor, which form a gradient of electrons in the cell membrane that mediates phosphorylation reactions to generate energy as adenosine triphosphate (ATP) (Lee et al., 2021). The bacterial oxidation reactions are generally

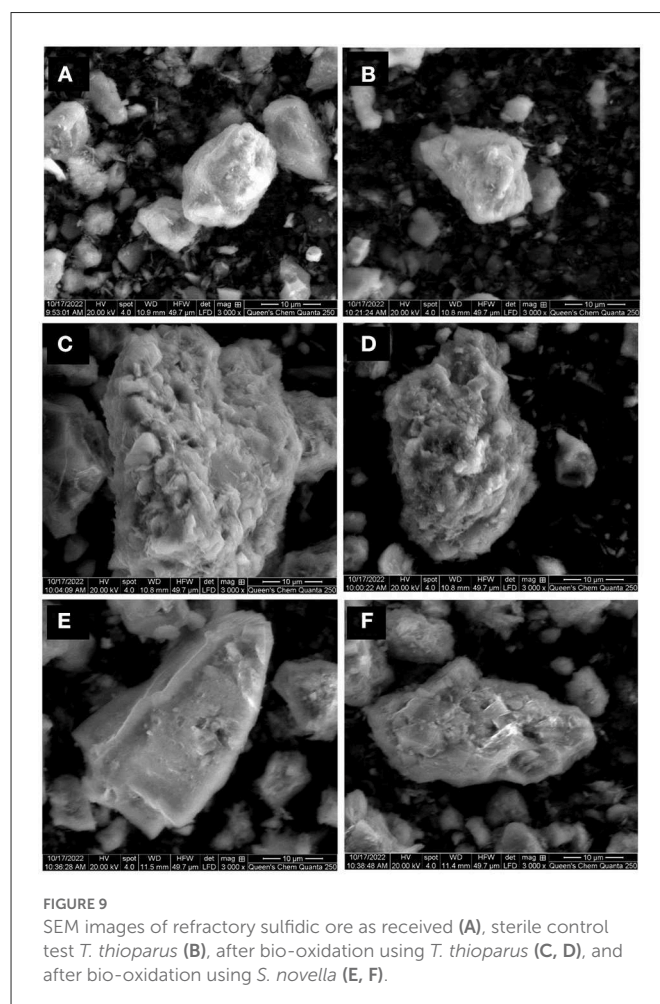


FIGURE 9 SEM images of refractory sulfidic ore as received (A), sterile control test *T. thioparus* (B), after bio-oxidation using *T. thioparus* (C, D), and after bio-oxidation using *S. novella* (E, F).

mediated by the catalytic action of extracellular enzymes secreted by the bacterium.

Regarding *T. thioparus*, the oxidation of reduced sulfur compounds proceeds through a chain of enzymatically mediated reactions, which might include the production of intermediate species such as sulfite and elemental sulfur with sulfate as final product. The oxidation with *T. thioparus* could involve the action

TABLE 5 Recent investigations including neutrophilic bacteria.

Microorganism	Sulfide source	S/L ratio (g/L)	Initial pH	Experimental time (days)	Results	References
<i>T. thioparus</i>	Mercury sulfide	2	7.0	77	<5% HgS oxidation	Vázquez-Rodríguez et al., 2015
Indigenous bacteria (<i>Rhizobiales</i> sp. and <i>Ralstonia</i> sp.)	Synthetic pyrite	2.3	6.8	30	20% sulfide oxidation	Percak-Dennett et al., 2017
<i>C. youngae</i>	Gold tailings	50	5	5	Increase in gold recovery by 6.1%	Purnomo et al., 2019
<i>T. thioparus</i>	Refractory sulfidic ore	10	6.6	10	27% sulfide oxidation	This study
<i>S. novella</i>	Refractory sulfidic ore	10	6.6	10	14% sulfide oxidation	This study

of enzymes including but not limited to thiosulfate reductase, APS (adenosine phosphosulfate) reductase, AMP (adenosine monophosphate), ADP (adenosine diphosphate) sulfurylase, ATP (adenosine triphosphate) sulfurylase, and adenylate kinase (Skarzynski and Szczepkowski, 1959; Chung et al., 1996; Kelly, 2006). Thus, sulfide oxidation could include an enzymatically mediated pathway that could consist of forming intermediate species, which can result subsequently in sulfate formation (Schippers et al., 1996, 2000). The oxidative mechanism of *S. novella* has been less investigated than *T. thioparus*. Still, it has been pointed out that it could have a mechanism encompassing soluble enzymes, similar to *T. thioparus*, and/or through multienzymes pathways oxidizing reduced sulfur species to sulfate (Yamanaka, 1996; Kappler et al., 2001).

The surface morphology of the sulfidic ore pre- and post-oxidative treatment was analyzed with SEM images (Figure 9). The surface of the as-received ore sample showed a smooth surface with no pits and cracks, as shown in Figure 9A. The surface of the sulfidic ore after the control test also displayed slight alterations, showing a similar morphology to the as-received sample. After the bio-oxidation pretreatment with both neutrophilic microorganisms, the surface of the sulfidic ore adopted a rougher appearance (Figures 9C–E). In the same way as S° SEM analysis in Figure 6, *T. thioparus* also led to greater biotransformation of the ore surface than *S. novella*, displaying a rougher surface and a more significant number of pits, which agreed with the higher oxidation rate obtained with *T. thioparus*. The pits identified after bio-oxidation with both strains have also been observed in bio-oxidation using acidophilic microorganisms (Azizitorghabeh et al., 2022). Nevertheless, the number of pits and degree of biotransformation was minor compared to the microorganisms commonly used in bio-oxidation at acidic pH (Li et al., 2016).

Literature comparison

Recent investigations comprising neutrophilic microorganisms are summarized in Table 5. Vázquez-Rodríguez et al. (2015) explored mercury (Hg) emission from sediment through the dissolution of mercury sulfide (HgS) employing *T. thioparus*. The study revealed that *T. thioparus* was able to use HgS as the sole energy source in

replacement of thiosulfate and the sulfate generation reached ca. 0.2 mM, which was 9–1,000 times greater than naturally occurring conditions. In another study, Percak-Dennett et al. (2017) evaluated pyrite oxidation employing indigenous bacteria isolated from pyrite-containing sediments identified as *Rhizobiales* species and *Ralstonia* species and obtained overall 20% sulfide oxidation after 30 days. More recently, Purnomo et al. (2019) implemented a 5-day bio-oxidation pretreatment using *C. youngae* to improve gold recovery from gold tailings containing 0.53% w/w total S. The outcome of the study was an enhancement in gold recovery by 6.1%. Compared with the recent studies, sulfide oxidation rates were similar in most investigations. However, higher solid-to-liquid ratios were chosen in this study, which could lead to higher sulfide oxidation rate considering lower solid-to-liquid ratios used in other investigations. In addition, the reaction time was 3 to 7 times lower than most other studies, showing higher potential for *T. thioparus* and *S. novella* under similar experimental conditions. Even though this study demonstrated that both bacteria are able to bio-transform S in different matrices, *T. thioparus* would be a better option to continue this investigation owing to its better performance compared to *S. novella*. There is still a more evident need for further research in the case of *S. novella* to improve its catalytic action to oxidize sulfur matrices.

Conclusion

This study showed the potential of *T. thioparus* and *S. novella* in oxidizing solid matrices. The bacterium *T. thioparus* displayed better oxidation in a culture medium with 4.48 g/L thiosulfate with an $OD_{600}(\max) = 0.6$. Contrarily, *S. novella* showed a better performance with a lower thiosulfate concentration (0.9 g/L) due to its metabolic characteristics, requiring organic reagents (1.5 g/L glucose and 0.3 g/L yeast extract) with a $OD_{600}(\max) = 0.5$. In bio-oxidation tests with pure S° , the microorganisms could use S° as a sole energy source, replacing the requirement of thiosulfate for its growth. The oxidation of S° with *T. thioparus* and *S. novella* reached 18% and 10% within 10 days. Subsequently, bio-oxidation using the low-grade refractory sulfidic ores containing 5.7% S in shake flasks showed 20.8 and 9.5% sulfide oxidation for *T. thioparus* and *S. novella*, respectively, within 10 days. The bio-oxidation results indicated that bacteria oxidize S° more easily than

S in the refractory ore. An extended experimental time of 30 days led to a ca. 25% sulfide oxidation with *T. thioparus* and 11.3% sulfide oxidation with *S. novella*. The implementation of a bioreactor positively increased sulfide oxidation with both microorganisms, reaching 27.2 and 14% for *T. thioparus* and *S. novella*, respectively. SEM analysis revealed that the bacterial action led to rougher surfaces and pits in the ore. The comparative study showed that *T. thioparus* and *S. novella* performed better than other bacteria using higher pulp densities and shortened experimental time. We demonstrated the potential of both microorganisms to oxidize refractory sulfidic ores, which can inspire future investigation toward an effective bio-oxidation process at higher pH with a net-zero target in the mining industry.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

GH: investigation, data curation, resources, and writing—original draft. HM: conceptualization, supervision, and writing—review and editing. AG: conceptualization, project administration, supervision, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsrma.2023.1102488/full#supplementary-material>

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